FEBS Letters 432 (1998) 40–44 FEBS 20584

Activation of NF-κB in human neutrophils during phagocytosis of bacteria independently of oxidant generation

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Received 29 June 1998

Abstract We have exposed human neutrophils to opsonized *Staphylococcus aureus* and used an electrophoretic mobility shift assay to show activation of the transcription factor NF-κB above basal levels. Activation was evident within 10 min and was increased with higher bacteria:neutrophil ratios. The neutrophil NADPH oxidase inhibitor diphenylene iodonium, catalase, and other oxidant scavengers did not inhibit NF-κB activation, and no activation was seen with added hydrogen peroxide. Oxidants produced during phagocytosis, therefore, are not involved in the activation mechanism.

Key words: Nuclear factor κB; Neutrophil; Phagocytosis; Hydrogen peroxide; *Staphylococcus aureus*

1. Introduction

Nuclear factor κB (NF-κB) is a ubiquitous transcription factor used extensively to regulate the expression of a range of inflammatory response genes [1–3]. Genes for tumor necrosis factor (TNF), interleukin (IL) 1β, interleukin-1 receptor antagonist, interleukin 8, granulocyte-macrophage colony stimulating factor, the interleukin 2 receptor and some adhesion molecules are among those with κB binding motifs in their 5′ regulatory region. Monocytes, macrophages and B and T lymphocytes exploit the versatility of the NF-κB system [1,2,4–6]. Neutrophils are terminally differentiated cells with a short circulating half-life. They have generally been considered only effector cells rather than orchestrators of the immune response. However, recent studies have shown that various soluble and particulate stimuli induce neutrophils to transcribe and translate genes that encode inflammatory mediators [7,8]. These include cytokines such as TNFα, IL-1β and IL-8 and interleukin-1 receptor antagonist [9–14] that in other cells are under the control of NF-κB. The potential for neutrophils to use NF-κB as a transcription factor has been identified by McDonald and coworkers [15,16] who demonstrated NF-κB activation by several soluble stimuli and by yeast particles. In contrast, Browning et al. [17] saw no activation. They concluded that this was due to insufficient amounts of NF-κB in the cytoplasm. NF-κB activation has also been observed in monocytes and endothelial cells exposed to micro-organisms or IgG aggregates [18,19].

NF-κB is a dimeric protein composed of members of the Rel family of proteins. The predominant form encountered is p50/RelA (p50/p65). NF-κB is sequestered in the cytoplasm in an inactive state by an inhibitor protein, I-κB. Activation requires dissociation of the transcription factor from its inhibitor and mobilisation to the nucleus, where it binds DNA containing the κB motif to regulate transcription [20]. There is substantial evidence that NF-κB is redox regulated [3,21,22]. Although the mechanism is not fully understood, activation appears to be influenced by the thiol oxidation state of the cell, and endogenous generation of reactive oxygen species following receptor-mediated activation has been proposed [23–25]. In some cell types, hydrogen peroxide is able to activate NF-κB directly [25–31].

Phagocytosis induces neutrophils to generate large amounts of hydrogen peroxide and other reactive oxygen species [32]. These oxidants are potential mediators of NF-κB activation. In this study, we have investigated whether NF-κB is activated in neutrophils during phagocytosis of *Staphylococcus aureus*, and used inhibitors and scavengers of oxidants to establish whether the mechanism involves oxidant generation.

2. Materials and methods

2.1. Reagents

Polyclonal rabbit antiserum raised against amino acids 3–19 of the amino-terminal domain of RelA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). κB probe, containing the NF-κB binding site from HIV-1 LTR [33], was purchased from Life Technologies (Gaithersburg, MD). rhTNFα was supplied by Dr. J. Michaelis (Peptide Technology, Australia). All cell culture materials were obtained from Life Technologies. Unless stated otherwise, biochemicals were purchased from Sigma (St. Louis, MO). Complete protease inhibitor, IFN-γ, and Klenow enzyme were obtained from Boehringer Mannheim (Germany), and Ficoll and Hypaque from Pharmacia Biotechnology (Uppsala, Sweden). α1-Antitrypsin was purified from human plasma [34]. Incubations were performed in phosphate buffer pH 7.4 containing 140 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5.6 mM glucose (Hanks' buffer).

S. aureus ATCC 27217 (502a) was obtained from the New Zealand Communicable Disease Centre, Porirua, New Zealand. The bacteria were cultured overnight in nutrient broth then opsonized with 10% plasma from the same donor as for the neutrophils. Zymosan was heated in phosphate buffer pH 7.4 containing 140 mM NaCl for 20 min at 90°C, followed by 20 min opsonisation at 37°C in Hanks' solution containing 33% plasma. Aggregated IgG was prepared as in [35].

2.2. Neutrophil isolation and stimulation

Neutrophils were isolated from blood from healthy human donors who had given informed consent, by Ficoll centrifugation, dextran sedimentation and hypotonic lysis [36]. At least 95% of the cell preparation consisted of neutrophils, with a viability of >95% as determined by trypan blue exclusion. Neutrophils (3×10^6/ml) were treated with the desired stimulus and incubated at 37°C with gentle rotation. *S. aureus* were incubated with neutrophils at a ratio of 20:1 [37]. Inhibitors were added to the cells 5 min prior to addition of the stimulus. Reactions were stopped by placing the cells on ice.

2.3. Electrophoretic mobility shift assay

Whole cell extracts were prepared by pelleting the cells, washing in Hanks' buffer and resuspending in ice-cold lysis buffer (20 mM

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PII: S0014-5793(98)00821-7

HEPES-KOH pH 7.5, 350 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 20% (v/v) glycerol, 1% (v/v) Nonidet P-40, 5 mM DTT, and 1.6 mg/ml Complete protease inhibitor) for 15 min on ice. Cell debris was pelleted at 14 000×*g* for 20 min at 4°C, and the supernatant used for analysis. Total protein was determined using a Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions using bovine serum albumin as standard.

The NF-κB DNA binding activity of extracts was determined by electrophoretic mobility shift assay (EMSA) according to the method of Zabel [33] except that Complete (1.5 mg/ml) was used in place of the protease inhibitors. Extract containing a standard amount of protein (usually 10 μg) was added to binding buffer to which KCl was supplemented to a final concentration of 100 mM. For specificity analysis, 100-fold excess of cold probe or 6 μg RelApAb was included in the binding reaction. Following a 10 min incubation on ice, ^{32}P-labelled probe was added and the solution left at room temperature for 20 min. Samples were loaded on to a 4% polyacrylamide gel, electrophoresed in 0.5×Tris-borate-EDTA, and exposed to film using an X-Omat intensifying screen (Kodak, USA). Band intensities were determined using video densitometry and compared using SigmaStat (Jandl Scientific) either by paired *t*-test or ANOVA.

3. Results

To investigate whether NF-κB is activated during bacterial ingestion, neutrophils were isolated from human blood and incubated with *S. aureus*. Whole cell extracts were subjected to EMSA with an oligonucleotide probe containing the consensus κB binding sequence. Whole cell extracts from Wurzburg T cells that had been treated with hydrogen peroxide to activate NF-κB [25] were used as a reference. The T cell extract gave two major bands in addition to free probe (Fig. 1, lane 1), the slower of which was identified as NF-κB-specific on the basis of it being outcompeted by cold probe (not shown) and supershifted by an antibody to the RelA subunit (Fig. 2A, lane 9). Extracts from unstimulated neutrophils (Fig. 1, lane 2) gave a band with a faster mobility than the reference. This band (marked with an arrow) increased in intensity when the neutrophils were incubated with opsonized *S. aureus* (lane 3).

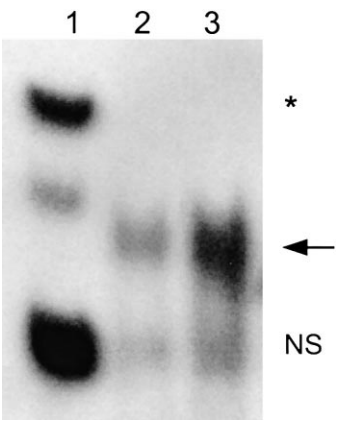

Fig. 1. EMSA detection of binding activity in whole cell extracts from neutrophils exposed to *S. aureus*, using an NF-κB-specific oligonucleotide probe. Lane 1: Reference NF-κB. Whole cell extract from Wurzburg T cells activated with 100 μM hydrogen peroxide as in [25]. Lane 2: Neutrophils plus *S. aureus* at a 1:20 ratio, placed on ice immediately after mixing. Lane 3: As in lane 2 but incubated for 30 min at 37°C. The faster moving κB-specific band in the neutrophil extracts is designated with an arrow and the band corresponding to the RelA/p50 complex in the T cell reference sample is shown by an asterisk. The other major band shown (NS) is not shifted by the cold probe and is non-specific. The lower section of the gel containing free probe is not shown.

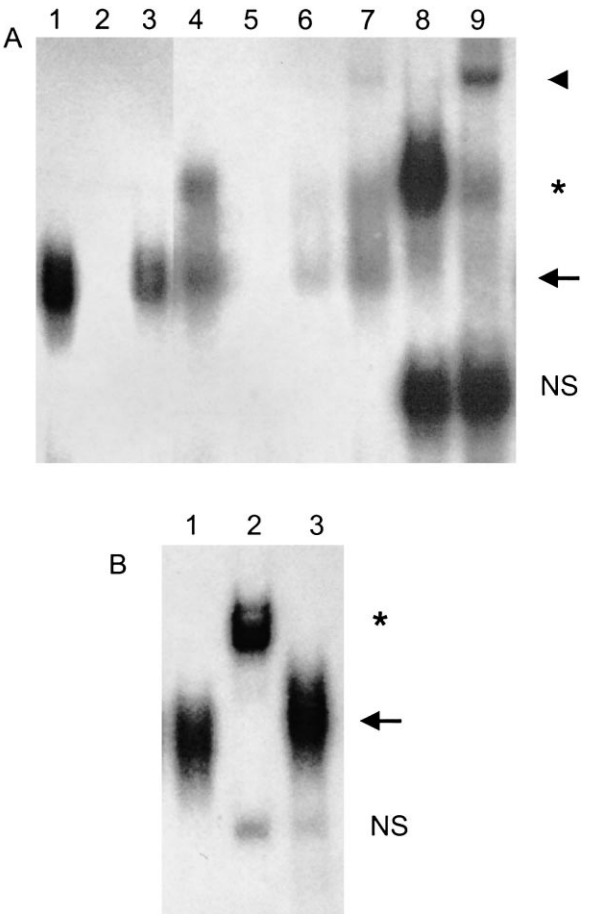

Fig. 2. A: Identification of neutrophil band as NF-κB. Lane 1: Extract from stimulated neutrophils. Lane 2: Extract from lane 1 with cold probe added. Lane 3: Extract from lane 1 with anti-RelA antibody added. Lanes 4–6: Same as lanes 1–3, but with α1-antitrypsin (3 mg/ml) added to the cell lysis buffer. Lane 7: Same as lane 6 but with 3 times as much protein loaded. Lane 8: Reference NF-κB from Wurzburg T cells plus hydrogen peroxide. Lane 9: Same as lane 8 with anti-RelA added. EMSA was performed as in Fig. 1, except that the cell extract was obtained from neutrophils following incubation with 5 mg/ml opsonized zymosan. Bands are identified as in Fig. 1, with the supershifted band marked by an arrowhead. B: Premixing of cell extracts from neutrophils and Wurzburg T cells to show degradation of NF-κB in the latter by neutrophil proteases. Conditions as in A. Lane 1: Neutrophil extract after activation with opsonized zymosan. Lane 2: Wurzburg cell extract. Lane 3: Both extracts mixed at room temperature for 10 min before performing EMSA.

To determine whether the binding activity in the neutrophil extracts represented NF-κB, several procedures were carried out. This band was outcompeted by cold probe (Fig. 2A, lane 2) and decreased in intensity when the RelA antibody was added (lane 3), although a supershift complex was not observed. To determine whether degradation by neutrophil proteases was responsible for the increase in mobility, α1-antitrypsin was included during preparation of cell extracts. This procedure was adopted after Franzoso et al. [38] showed that NF-κB in extracts in subclones of U937 promyelocytic cells underwent proteolysis during processing to give a fast band. This was not prevented by standard inhibitor cocktails, but high concentrations of the serine protease inhibitor α1-antitrypsin gave normal mobility. Addition of 3 mg/ml α1-antitrypsin to neutrophil extracts gave two diffuse electrophoretic

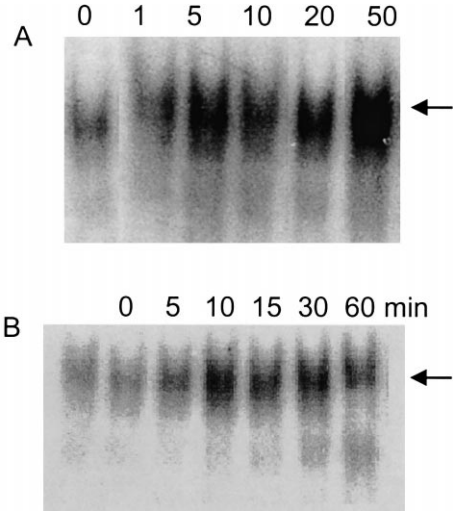

Fig. 3. EMSA of neutrophil extracts showing NF-κB activation with opsonised *S. aureus*. A: Dependence on number of bacteria added. Assays were performed 30 min after mixing. Ratios of bacteria:neutrophils are shown above each lane. B: Time course of activation with bacteria:neutrophil ratio of 20:1. The first lane shows background activation with no bacteria after 30 min. Incubation times in minutes are shown above the other lanes. For the zero time sample, cells were cooled on ice immediately after mixing. In each case, the results are from one of two experiments performed with different neutrophil donors. Both gave similar results. The portion of the gel showing the neutrophil NF-κB band is shown.

bands (Fig. 2A, lane 4), one in the same position as in the control sample and the other with the same mobility as T cell NF-κB (lane 8). Both bands were outcompeted by cold probe (lane 5) and decreased in intensity by the RelA antibody (lane 6). With a higher loading of the antibody-treated extract, a faint supershift complex was observed (lane 7) with the same mobility as supershifted T cell NF-κB (lane 9).

Confirmation of proteolytic degradation was obtained by co-incubating extracts from activated T cells and neutrophils for a brief period (Fig. 2B). Only one NF-κB band with similar mobility to neutrophil NF-κB was observed. These results indicate that although proteolysis in neutrophil extracts leads to a faster electrophoretic band, this band is nonetheless specific for NF-κB. Since blocking proteolysis required large amounts of α1-antitrypsin, and even then was only partially effective, subsequent experiments were performed using only the standard inhibitor cocktail and changes in the faster band were assessed.

We always observed some NF-κB activation in unstimulated neutrophils. This was already present in the isolated cells, as further incubation did not increase the level of activity. Although the basal level of activity varied between preparations and in some cases obscured any effect of the bacteria, the results of all the experiments performed showed a significant increase in NF-κB binding: 10–60 min exposure to *S aureus* gave a 1.40-fold increase in NF-κB band intensity above control, S.E. = 0.06, $n = 34$, $P < 0.001$. Activation of NF-κB depended on the number of bacteria added. It was scarcely above background with a 1:1 *S. aureus* to neutrophil ratio, but increased progressively with ratios of 5:1 to 50:1 (Fig. 3A). Most of the activation occurred during the early stages of a 1 h incubation (Fig. 3B).

Since phagocytic stimuli induce neutrophils to undergo an oxidative burst, we tested whether oxidants were responsible for the NF-κB activation in neutrophils incubated with *S. aureus*. The addition of diphenylene iodonium, which inhibits the NADPH oxidase and gives less than 5% normal superoxide production [37], or catalase to scavenge hydrogen peroxide, had no effect on the degree of stimulation (Fig. 4). In other experiments, addition of superoxide dismutase, or methionine to scavenge myeloperoxidase-derived hypochlorous acid, gave no inhibition. Consistent with the lack of inhibition by oxidant scavengers, exogenous hydrogen peroxide added to neutrophils gave no NF-κB activation over a 1 h period (Fig. 4).

To determine whether phagocytosing neutrophils release a stable factor that could activate NF-κB in neighboring cells, neutrophils were incubated with opsonized zymosan for 30 min, then the supernatant was removed and added to fresh neutrophils. Analysis by EMSA showed a strong NF-κB band in the cells exposed to zymosan, but activation in the fresh neutrophils was no higher than in cells incubated in Hanks' buffer alone (not shown).

Other stimuli were tested. Increased binding to the probe was observed when the neutrophils were incubated with opsonized zymosan (5 mg/ml) or with aggregated IgG (0.5 mg/ml) suggesting NF-κB activation. However, cell extracts prepared immediately after adding these phagocytic stimuli showed increased DNA binding activity, so whether this represents true activation is uncertain. TNFα and LPS gave small increases in binding activity, observed after 1 h. No increase over unstimulated cells was seen with phorbol myristate acetate (PMA), the calcium ionophore A23187, interferon γ, granulocyte colony stimulating factor or f-Met-Leu-Phe. Incubation of the cells in RPMI as against Hanks' solution gave

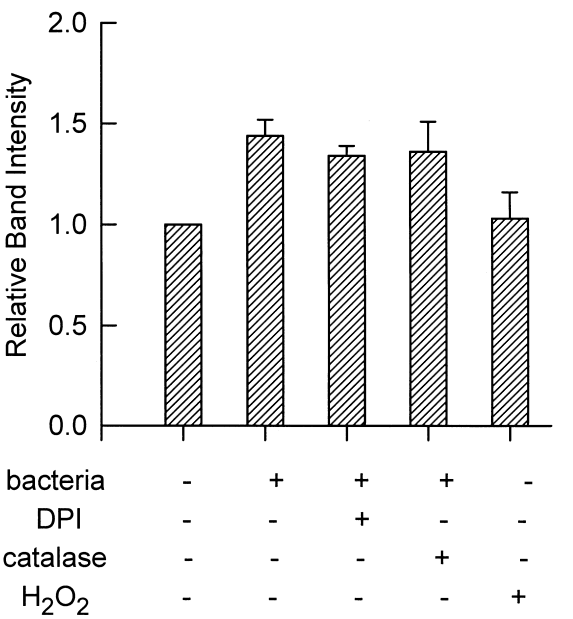

Fig. 4. Effect of oxidant inhibitors and scavengers on neutrophil NF-κB activation by *S. aureus* determined by video densitometry. Neutrophils (3×10^6) were incubated for 30 min with *S. aureus* (6×10^7) and, where indicated, 10 μM diphenylene iodonium (DPI), 20 μg/ml catalase or 100 μM hydrogen peroxide. Results are means ± S.E.M. of five experiments. Band intensities were not significantly different in cells exposed to bacteria with or without DPI or catalase, and all were significantly higher than the unstimulated control ($P < 0.05$, ANOVA). Hydrogen peroxide treatment was not significantly different from controls.

no apparent difference in the degree of activation observed with any stimulus (results not shown).

4. Discussion

We have shown that NF-κB can be activated in neutrophils following phagocytosis of *S. aureus*. Activation, detected by EMSA with a κB-specific probe, was evident within 10 min. It was more pronounced at the higher bacteria to neutrophil ratios. This is the first report of NF-κB activation in neutrophils by live bacteria. Our findings extend those of McDonald et al. [16] who saw activation in neutrophils exposed to heat-killed yeast. The modest activation we saw with TNFα or LPS but not other soluble stimuli also agrees more with the findings of McDonald et al. [15], rather than with Browning et al. [17] who reported no activation. In the former study the p50 and RelA subunits were shown to be present in neutrophils. Initially they were predominantly cytoplasmic, but on stimulation, degradation of I-κB and migration into the nucleus was observed.

The increase in NF-κB activity over background caused by bacterial ingestion was significant but relatively modest. This may be a consequence of some cell preparations already showing substantial activity rather than necessarily indicating a low response to the stimulus. Alternatively, only a subset of the cells may respond. The activity observed in extracts from freshly isolated neutrophils could represent constitutive NF-κB in circulating neutrophils. However, others found less background activity in extracts of neutrophils lysed by nitrogen cavitation [15], implying that it mainly occurs during processing.

An unusual finding was that the neutrophil NF-κB complex observed by EMSA had a faster mobility than the p50/RelA complex in T cell extracts. Our results with added α1-antitrypsin and with mixing T cell and neutrophil extracts show that this was due to rapid proteolytic cleavage by a constituent of the neutrophil extract. Similar findings have been reported by Franzoso et al. [38] for some U937 subclones. They inhibited degradation with α1-antitrypsin or di-isopropylfluorophosphate (DFP), and McDonald et al. [15,16] routinely used DFP when preparing neutrophil extracts. Since antibody binding is abolished during proteolysis, it appears that the C-terminus containing the activation domain of RelA is cleaved. The concentrations of α1-antitrypsin required to partially inhibit degradation are higher than would be needed to inhibit the major neutrophil serine proteases, elastase and cathepsin G. Specific inhibitors of these enzymes were also ineffective (not shown). This suggests that neutrophils contain a serine protease that has a high reactivity towards RelA. If this protease were present in the cytoplasm, it could abrogate any effects of NF-κB activation. However, it is more likely to be one of the granule proteases released during processing.

Since oxidants have been shown to activate NF-κB activation in other cells, the large amounts of superoxide, hydrogen peroxide and hypochlorous acid generated during phagocytosis could provide the trigger for activation in neutrophils. We found that inhibiting the oxidative burst with diphenylene iodonium or adding oxidant scavengers did not inhibit activation. Furthermore, hydrogen peroxide added exogenously did not activate the cells. There is also no correlation between the extent to which soluble stimuli induce superoxide production and their ability to activate NF-κB. We conclude, therefore, that activation of NF-κB in neutrophils does not occur by an oxidative mechanism. The situation appears to be different with alveolar macrophages, which showed catalase-sensitive NF-κB activation by PMA and activation by hydrogen peroxide [31].

It is generally considered that once neutrophils have phagocytosed bacteria, they have performed their function and are destined for death. Indeed, a few hours after stimulation they show changes associated with apoptosis [39,40]. It is intriguing, therefore, that a transcription factor is activated in these cells. We reasoned that it might be advantageous for phagocytosing cells to release a factor that could regulate transcription in adjacent cells, but no soluble activator of NF-κB was detected. Inflammatory molecules such as TNFα and IL-8 are transcriptionally regulated by NF-κB and are synthesised in neutrophils following phagocytosis [9,10]. Whether NF-κB activates transcription in cells containing ingested bacteria, and if so for what purpose, are questions that require further investigation.

Acknowledgements: This work was supported by the Health Research Council of New Zealand. We are grateful to K. McPherson for assistance with the gel shift assays and Dr. M. Vissers for providing the IgG and α1-antitrypsin.

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